TITLE: **Detection, Isolation and Identification of *E. coli* O157:H7 from Foods and Environmental Sources Using the Pathatrix® Recirculating Immunomagnetic Separation (RIMS) System**

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Sign off sheet for: Procedures, Equipment, Test Methods, Work Instructions

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Procedure Outline

1.0 Introduction

1.1.1 Purpose and Background

The purpose of this method is to detect, isolate and identify *Escherichia coli* O157:H7 and *E. coli* O157:NM from foods and environmental samples, including water, soil, sediment, feces, and swabs, by recirculating the entire sample and pre-enrichment broth over antibody-coated paramagnetic beads (immunomagnetic recovery and concentration), and using selective media, antisera, biochemical analysis, and PCR for confirmation.

Pathatrix® is a microbial capture and concentration system that can utilize an entire food sample homogenate or enrichment culture by re-circulating the mixture over antibody-coated paramagnetic beads to selectively and simultaneously capture and concentrate the organisms. After continuous re-

circulation of the sample through the system, residue and food debris are removed by washing the beads. Aliquots of beads are plated and streaked directly onto selective agar for isolation, and extracted for screening real-time PCR to rapidly identify the presence of the organisms. The Pathatrix has been validated and AOAC-approved for the *E. coli* O157:H7, Salmonella and Listeria in foods.

This method is qualitative, not quantitative, and results should be reported as "*E. coli* O157 detected" (positive) or "*E. coli* O157 not detected" (negative). Negative results may be available after 24 hours, positive results at four days.

1.1.2 Limits of Detection

This method has been validated to detect <1 colony-forming unit per gram (cfu/g) from 150 g samples of spinach and romaine lettuce, < 6 cfu per sample from 100 ml samples of surface water, and <9 cfu per sample in a modified Moore swab (5).

1.2 Safety

E. coli O157:H7/NM is a human pathogen with a low infectious dose: ingestion of 10 – 100 cells can cause disease. The use of gloves and eye protection is mandatory and all work surfaces must be disinfected prior to and immediately after use. Laboratory personnel must follow CDC guidelines for manipulating Biosafety Level II pathogens. A Class II laminar flow biosafety cabinet is recommended for activities with potential for producing aerosols of pathogens. All available Material Safety Data Sheets (MSDS) obtained from the manufacturer for the media, chemicals, reagents and microorganisms used in the analysis are kept in the appropriate laboratory areas. The personnel who will handle the materials should read all MSDS sheets. All workers in the laboratory should abide by all federal, state, local and laboratory safety guidelines and rules where they apply when using this method

1.3 Quality Control

a. Every batch of all media is tested for sterility and support of target organisms.
b. CHROMagar™ O157 plates have a shelf life of 1 week.
c. All mBPWp and BPW media must be pre-warmed to 37-42ºC prior to use.
d. The ATCC #43888 strain of *E. coli* O157:H7 is used in this procedure as a positive control (see below). This strain does not contain stx 1 or 2 genes, but does contain the uidA mutation, as do other strains of *E. coli* O157.
e. The ATCC #25922 commensal strain of *E. coli* is used in this procedure as a control for sorbitol fermentation and MUG (see 1.4.4 below).

1.4 Equipment, Materials, Supplies, Media. Reagents, Control Cultures

1.4.1 Equipment

a. Pathatrix® workstation (Matrix MicroScience Inc, Golden, Colorado). The workstation houses 5 independent units, each has its own incubation pot with heating element, motorized pump head, magnet and control panel.
b. Balance, sensitivity 0.1 g.
c. Shaking incubator, 42°C, ± 0.5°C.
d. Vortex
e. Incubator, static, 37°C, ±2°C.
f. Micropipettors (Gilson or equivalent) to deliver 20 – 200 µl.
g. Mechanical pipetting device that accommodates 1, 5, 10 and 25 ml pipettes.
h. Vitek 2 Compact Biochemical Analysis System.
i. Waterbath, 37-42°C ±2°C.
j. Hand-held long wave UV light source.
k. Digital thermometer, -10°C to 100°C.
l. Optional: Stomacher™ 400 (Topac Instruments, Hingham, MA).
m. Optional: Blender, 1 L capacity

1.4.2 Materials and Supplies

a. Pre-assembled, sterile, individually packed Pathatrix® capture phase tubing units and collection tubes.
b. Whirl-pak™ 55 oz. filter bags, and 24 oz. Stand-Up bags.
c. Sterile graduated cylinders, 250 ml and 500 ml ±2%.
d. Sterile forceps, spoons, scoops, knives, scalpels and scissors (depending on sample type).
e. Sterile aerosol-resistant micropipette tips, 20 µl and 200 ul.
f. Sterile inoculating loops and needles.
g. Sterile pipettes, 1, 5, 10 and 25 ml.
h. Sterile disposable 12 X 75 mm polystyrene tubes with cap.
i. Whirl-pak™ bag racks.
j. Filter paper discs, 7 cm diameter (Whatman #1 or equivalent).
k. Optional: Poultry rinse bags.
1. CryoBeads™ (Hardy Diagnostics).

1.4.3 Media and Reagents

a. Pathatrix® PE50SF *E. coli* O157 Same-Day Test Antibody-coated beads (Matrix MicroScience, Golden, CO).
b. Buffer Peptone Water [Media Card #M-11, BAM M192].
c. Selective agar plates, TC-SMAC [Media Card #M-54, BAM M139] and CHROMagar™ for *E. coli* O157 (DRG, New Jersey) [Media Card #M-14].
d. Modified Buffered Peptone Water with sodium pyruvate (mBPWp) [Media Card #M-35].
e. 2X Modified Buffered Peptone with sodium pyruvate (2X mBPWp) [Media Card #M-35].
f. Levine's eosin-methylene-blue (EMB) agar plates [Media Card #M-21, BAM M80].
g. Trypticase soy agar with yeast extract (TSA-YE) plates [Media Card #M-24, BAM M59].

h. 4-methyl-umbelliferyl-β-d-glucuronide (MUG) discs (Hardy Diagnostics).
i. Oxidase Reagent [Reagent Card #R-23, BAM R-54]
j. BactiCard® E. coli or Kovacs Reagent (Remel, Lenexa, KS).
k. RIM™ E. coli O157:H7 Latex Agglutination test (Remel, Lenexa, KS).
1. Motility Test Medium (MTM) [Media Card #M-38, BAM M103].
m. Heart Infusion Broth (HIB) [Media Card #M-7, BAM M60].
n. Heart Infusion Agar (HIA) [Media Card #M-24, BAM M59].
o. Vitek 2 Compact Gram Negative (GN) card (BioMerieux).
p. Trypticase soy agar (TSA) plates [Media Card #M-60, BAM M152].

1.4.4 Control Cultures

a. *E. coli* O157:H7 ATCC# 43888, (MUG-, sorbitol-, serology+, uidAm+, stx -).
b. Commensal *E. coli* ATCC# 25922 (MUG +, sorbitol +, serology -, uidAm-, stx-)

1.5 Sample Processing and Pre-enrichment

1.5.1 Sample Receipt

a. See SOPs QA-306 for Chain of Custody requirements; QA-308 for Preparing Sample Description form; GM-506 for General Sample Check-In. Samples should arrive in the appropriate cooler containers with blue ice, NOT wet ice. Immediately after first opening external container take temperature of interior with sample digital thermometer and proceed as per SOPs.

1.5.2 Leafy Green Vegetables

a. Composite no more than five heads of leafy greens per sample. Weigh out 150 grams per composite sample (i.e., 50 grams from three heads, etc.) into a 55 oz Whirlpak™ filter bag.
b. Add 300 ml of pre-warmed 1X mBPWp. Fold over top of bag to seal, and palpate bag to mix thoroughly.

1.5.3 Water

a. This step includes water from any source, including surface water from streams, ditches, rivers, ponds, reservoirs, irrigation, and well water. Measure with sterile graduate, or weigh out into a 55 oz Whirlpak™ filter bag, 100ml of water sample. Avoid rocks, sticks, leaves, and debris that may puncture the bag.
b. Add 100 ml of pre-warmed 2X mBPWp. Fold over top of bag to seal, and palpate bag to mix thoroughly.

1.5.4 Solid Samples

a. This step is for solid samples of any kind, including solid foods (ground meats, ready-to-eat food, shellfish, etc), feces, sediments from surface water sources, and soil. Weigh out 25 g of sample into a 55 oz Whirlpak™ filter bag.

b. Add 225 ml pre-warmed 1X mBPWp and fold over top to seal. Palpate bag to mix thoroughly (feces, sediment, soils, soft foods). Some solid foods may require more vigorous mixing, such as shellfish. A Stomacher or blender may be used. If using a stomacher, double bag the sample in the Whirlpak bag into a Poultry Rinse bag and stomach the mixture for 30 seconds at medium speed. If a blender is used, sample may be weighed into the blender, mBPWp added, and blended for 1 min. Transfer to a 55 oz filter bag and seal.

1.5.5 Swabs

1.5.5.1 Modified Moore Swabs

a. Modified Moore swabs are gauze mops that have been immersed in flowing surface or sewage water for 4 – 6 days. They are collected into a Whirlpak™ bag in the field, 1 per bag per location of deployment. Remove modified Moore swab from original container and suspend with sterile forceps above an open 55 oz Whirlpak™ filter bag. With sterile scissors, clip the legs of the mop free from the knob and plastic tie into the bag.
b. Add 250 ml pre-warmed 1X mBPWp to the filter bag, and fold over the top several times to seal. Mix thoroughly by palpating the bag.

1.5.5.2 Environmental swabs

a. Many types of environmental swabs are available depending on the application: forensic evidence of fibers, for chemicals and for biologicals. For recovery of viable pathogenic bacteria from most surfaces, the SolarCult® sponge in sterile buffered peptone water (Solar Biologicals, Inc, Ogdensburg, NY) has proven the most efficacious in environmental studies. Up to four swabs may be composited into a single Whirlpak™ bag at the collection site. Transfer the swabs with sterile forceps to a 55 oz Whirlpak filter bag.
b. Add 250 ml pre-warmed 1X mBPWp to the filter bag, and seal by turning the wire tabs down several times. Mix thoroughly by palpating the bag.

1.5.6 Pre-enrichment

a. Place 55 oz Whirlpak™ filter bags with samples and broth into baskets in a shaking incubator, and incubate with constant shaking at 42°C for exactly 5 hours. Stagger batches at ½ - 1 hour intervals as needed for the Pathatrix®.
b. Remove from shaking incubator. Transfer each sample to a 24 oz Stand-Up Whirlpak™ bag pouring from the "clean" side of the filter and adjusting the filter to

avoid large particulates. Some samples containing large solids, particularly solid feces, may need to be filtered a second time.

1.6 Microbial Capture and Concentration with Pathatrix®

1.6.1 RIMS

a. Turn on Pathatrix® workstation approximately ½ hour before needed. Set run parameters at 37°C, 30 minutes running time, and pump speed at 02 (default setting). Pre-warm the pots to 37°C.
b. After 5 hours incubation place the Stand-Up bags containing sample homogenate and pre-enrichment broth into the thermally controlled pot (Figure 1). Unroll wire closure and open bag carefully.
c. Aseptically remove the sterile, disposable Pathatrix® tubing and capture kit from the bag, place the pot lid inside the Stand-Up bag and gently push the lid into place, to the surface of the broth in the bag (Figure 2).
d. Secure the capture phase cartridge within the magnet ensuring that the phase collar is located on the right hand side of the magnet (Figure 3). Seal mouth of Stand-Up bag around base of tubing with bag wires to prevent aerosols.
e. Load the inlet tubing into the pump head and take care when closing the pump head not to trap the tubing (Figure 4).
f. Vortex the vial of antibody coated beads for a minimum of 10 seconds prior to dispensing.
g. Aseptically add 50 µl of the beads into the outlet tube (Figure 5).
h. Push the SET button and make sure run parameters are correct: 37°C, 30 minutes, pump speed 02.
i. Press the RUN button; recirculation will start immediately. Carefully maneuver the pot lid down into broth to eliminate air bubbles in the circulation tubing.

1.6.2 Washing and elution

a. Remove wash/ collection tube from sterile pack and aseptically fill to the line with approximately 100 ml pre-warmed BPW. Place in wash vessel magnet/ holder (Figure 6).
b. When 30 minute run is over, separate tubing at "inline connector" and attach the "inline connector" to the wash vessel lid (Figure 7).
c. Press RUN on the keypad to initiate wash step (Figure 8).
d. Press RUN to stop the pump just as the last of the wash BPW enters tube and before air enters phase.
e. Block the outlet tubing using a plastic clamp (Figure 9).
f. Disconnect the "inline connector" from the wash vessel lid and attach a 5 ml transfer pipette bulb (provided with kit) to the connector.
g. Open the pump head, releasing the pressure, and detach the inlet tubing from the phase end and allow the liquid to drain into the Stand-Up bag.
h. Remove tubing from pump head and place in Stand-Up bag to prevent dripping.

i. Attach the wash/collection vessel to the phase and carefully remove the phase with the wash vessel and pipette bulb from the magnet (Figure 10), and place the whole set to the magnet rack (provided) (Figure 11).
j. Unclamp the tubing and gently flush the wash buffer over the phase at least 5 times using the transfer pipette bulb. Finally, fully depress the pipette bulb to expel all wash buffer into the wash vessel.
k. Discard the lid/phase/tubing/bulb and replace with the flat lid provided.
1. Allow the wash vessel with beads to stand at room temperature for 5-10 minutes for the beads to be drawn to the magnet (Figure 12).
m. Without disturbing the beads on the side of the tube, aseptically remove the lid, and remove all residual wash buffer with sterile pipette (5, 10 or 25 ml) (Figure 13). Resuspend beads with 80 µl fresh BPW.
n. The sample is now ready for use with direct plating, real-time PCR and other detection methods.

1.7 Isolation, Identification and Confirmation

1.7.1 Day 1: Selective Media

a. With micropipettor, deliver 30 ul of washed beads @ to each of two plates, either 2 CHROMagar plates, or 1 CHROMagar and one TC-SMAC plate. Reserve the remaining 20 ul for screening real-time PCR (See SOP PCR-1000, Jinneman et al, 2003). If PCR is not going to be done the same day, freeze at –30-80^{o}C.
b. Streak plates for isolation, changing loops after the first section is spread. Incubate at 37^{o}C for 24 hours. The time (24 hours) is critical for development of the reddish pigment that characterizes *E. coli* O157 on CHROMagar and to allow slow sorbitol fermenters to develop color on TC-SMAC.

1.7.2 Day 2: Differential Media

a. At 24 hours examine plates for typical colonies (Figure 14). Record no growth (NG), typical (T) or atypical (AT) characteristics. Pick a single typical, well-isolated colony to EMB and TSA-YE plates using the same needle, discard needle and streak for isolation with new needle or loop. Pick a minimum of four colonies per selective plate if there are that many, more if warranted. If there are numerous typical colonies, two picks from the same sample may be streaked on half-plates of EMB and TSA-YE.
b. Place a MUG disc on the heaviest portion of the TSA-YE, and gently tap into place.
c. Incubate plates 18 – 24 hours at 37^{o}C.

1.7.3 Day 3: Oxidase, Serology, Indole and Vitek 2

a. Record T or AT for EMB growth characteristics (Figure 15). Read MUG discs under UV light and record.

b. Using colonies from the TSA-YE plate for isolates that are EMB+ and MUG-:
   1) transfer growth to a 7 cm disc of filter paper. Apply oxidase reagent, read after 1 min. and record.
   2) transfer growth to a RIM™ E. coli O157:H7 card per manufactures instructions. Read immediately for agglutination and record.
   3) transfer growth to a BactiCard® E. coli per manufacturer's instructions, or apply Kovac's reagent to an isolated colony and read for color development and record.

c. For isolates that are oxidase-, serology+ and indole- inoculate a Vitek 2 GN card per manufacturer's instructions from TSA-YE plate. Place in Vitek 2 for biochemical identification.

d. For each of the above isolates inoculate one motility deep, one small tube HIA for PFGE, and one small tube HIB for PCR confirmation.

1.7.4 Day 4 Motility, PCR Confirmation

a. Read and record Vitek identification and motility reaction. Refer isolates that are identified as *E. coli* O157 by the Vitek for confirmation by real-time PCR (see PCR-1000 or Jinneman, et al, 2003.) Hold HIA tube for PFGE pending PCR results.

b. Following PCR, report isolates that are *uidA* mutation positive as "*E. coli* O157:H7 detected" or "E. coli O157:NM detected" depending on motility reaction. Record

1.8 Isolate Library Storage

a. Selected representative isolates from each outbreak and all non-O157 stx+ *E. coli* (STEC) are saved in the Isolate Library. Inoculate one plate of TSA, incubate 24 hours, and follow Isolate Storage and Freezing protocol, FM-615.

1.9 References

1. BAM Bacteriological Analytical Manual, Revision January 2001 http://vm.cfsan.fda.gov/~ebam/bam-toc.html.
2. USDA/FSIS Microbiology Laboratory Guidebook http://www.fsis.usda.gov/Science/Microbiological_Lab_Guidebook/index.asp.
3. Pathatrix user manual, Matrix MicroScience, Inc. http://www.matrixmsci.com/pathatrix.htm.
4. **Vitek 2 Compact User Manual**. 2004. BioMerieux, Inc, Durham, NC.
5. **Himathongkham, S, ML Dodd, JK Yee, DK Lau, RG Bryant, AS Badoiu, HK Lau, LS Guthertz, and LK Crawford-Miksza**. 2007. Optimal enrichment conditions and recirculating immunomagnetic separation (RIMS) for enhanced detection and recovery of low levels of *Escherichia coli* O157:H7 from fresh leafy produce and surface water, *J. Food Protect.*, submitted.
6. **Jinneman, KC, KJ Yoshitomi, SD Weagant**. 2003. Multiplex real-time PCR method to

identify Shiga toxin genes stx1 and stx2 and Escherichia coli O157:H7/H- serotype. *Appl. Environ Microbiol*. **69:**6327-33.

Figure 1

Figure 2

Figure 3

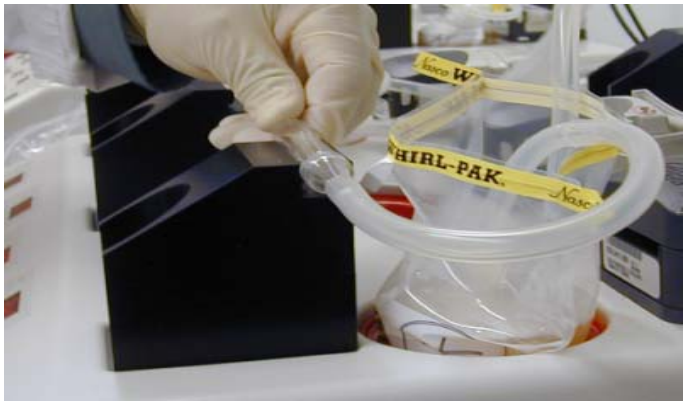


Figure 4

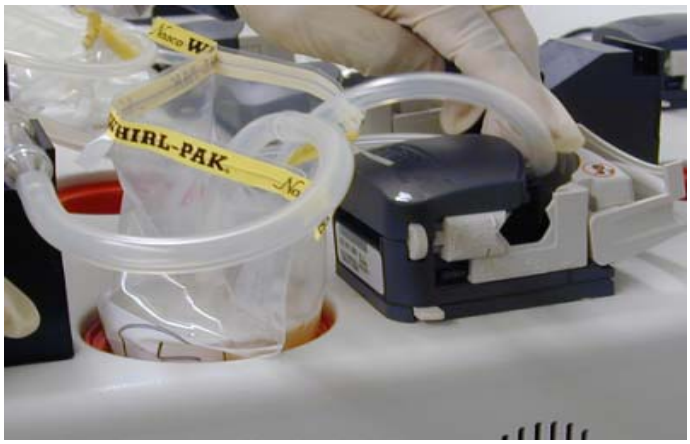
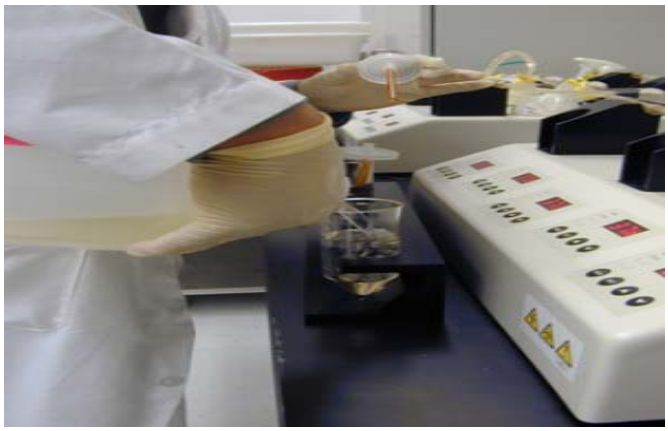


Figure 5

Figure 6



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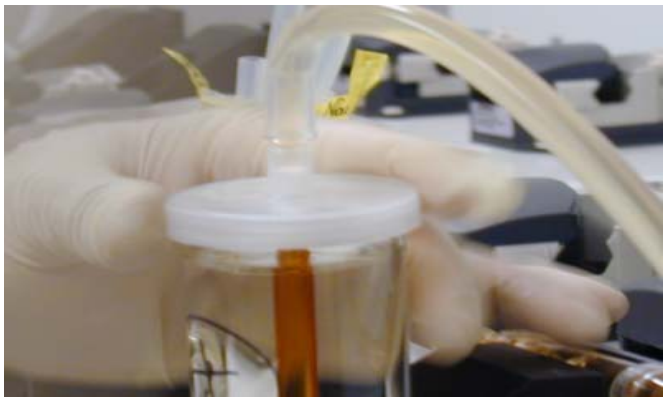

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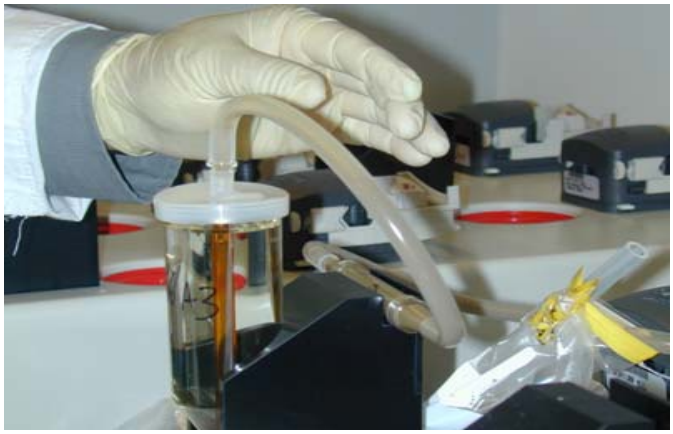

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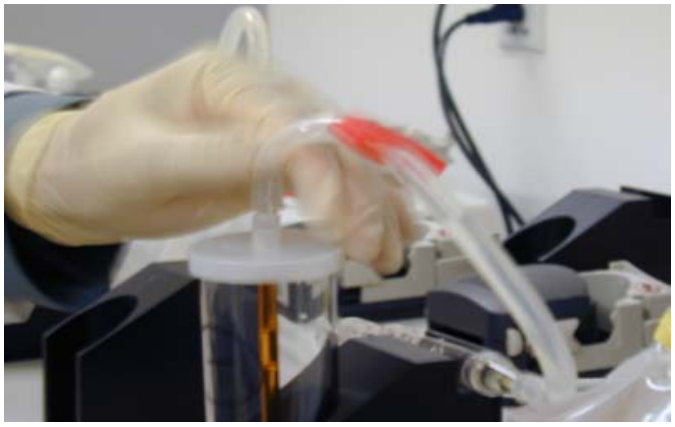

Figure 10

Figure 11

Figure 12

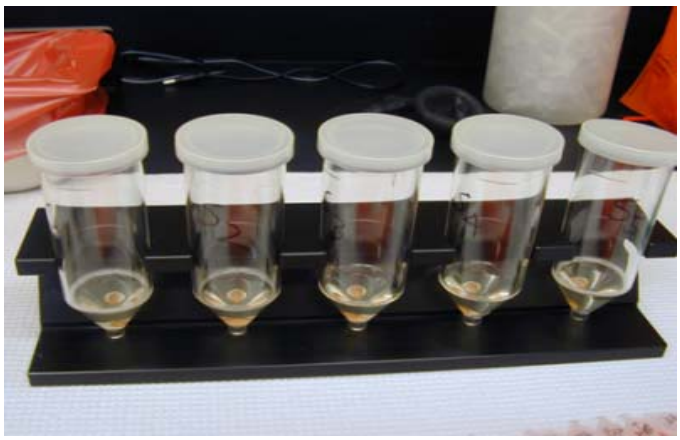

Figure 13

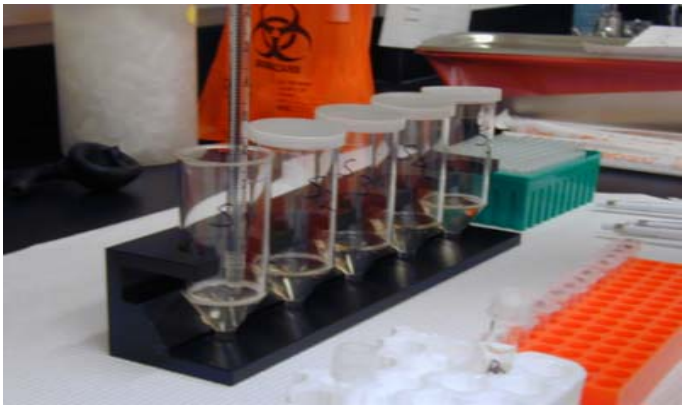

Figure 14

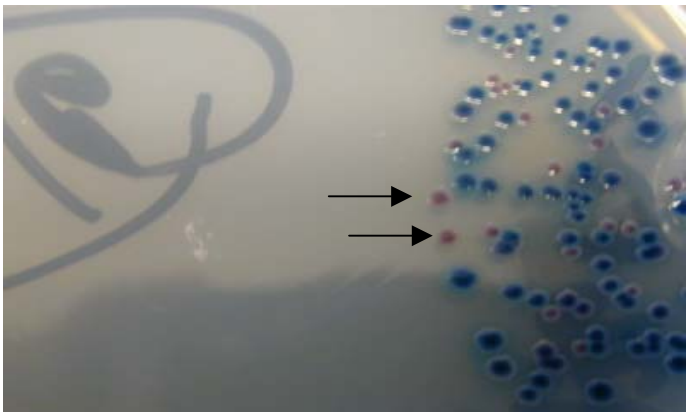

Figure 15